THE LOCATION OF THREE COLLAGEN TYPES IN SKELETAL MUSCLE

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1. Introduction

Collagen is the major supporting tissue of skeletal muscles and these collagenous tissues have been classified on anatomical and histological grounds. Thus, the epimysium forms the outer sheath of the muscle, the perimysium surrounds the muscle fibre bundles and the endomysium surrounds each individual muscle fibre [1]. Histologically the peri- and endomysium are composed of fine fibres and are often referred to as reticulin. Further, the endomysium appears from electron microscope studies to be mainly an amorphous basement membrane structure [2].

The existence of several chemically and genetically distinct collagens has now been established [3]. Skin, tendon and bone consist mainly of Type I collagen, hyaline cartilage Type II, foetal skin and blood vessels mainly Type III and basement membrane Type IV collagen. Our recent chemical studies have indicated the presence of collagen Types I, III and a third form, believed to be Type IV, in bovine muscle [4]. It is possible that there is a correlation between the histological classification and these genetic types of collagen. In this study we have therefore attempted firstly, to confirm the identity of the 'Type IV' as basement membrane and secondly to determine the tissue-specific location of these collagens by immunofluorescence using antibodies that react specifically with the three polymorphic forms of collagen.

2. Materials and methods

2.1. *Preparation of antigens*

Collagen Types I and III were prepared from foetal calf skin following pepsin digestion and fractional precipitation with sodium chloride [5,6]. The precipitates obtained were reprecipitated and shown to be a single type of collagen by SDS–polyacrylamide gel electrophoresis [6].

The Type IV collagen was obtained from human placenta, i.e., the chorionic villi of the cotyledons, dissected free from the amniotic foetal membrane [4]. Following extensive washing and pepsin digestion, the Types I and III were precipitated with 0.9 M NaCl. The supernatant contained the Type IV collagen which was then purified by precipitation from 1 M NaCl, 50 mM Tris–HCl, at pH 7.5, with 4.0 M NaCl. The purity of the collagen was assessed by SDS–gel electrophoresis.

The precipitates were dissolved in 0.5 M acetic acid (2 mg/ml) dialysed against water, lyophilized and stored at –20°C.

2.2. *Preparation of antisera*

Antibodies to pepsin extracted Type I and III collagen from foetal calf and 'Type IV' from human placenta were raised in New Zealand white rabbits. The antigens (2 mg/ml) in water were initially emulsified in an equal volume of Freund's complete adjuvant and in Freund's incomplete adjuvant for subsequent injections. Injections (0.5 ml) were given at four subcutaneous sites at fortnightly intervals. Detectable antibodies appeared on average after 4–6 injections.

Immunoadsorbent columns were used to remove unspecific antibodies from high titer antisera. Types I and III foetal calf skin collagen were attached to Sepharose 4B by the method of March et al. [7]. Columns were equilibrated in 0.02 M sodium dihy-

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drogen orthophosphate, 0.15 M NaCl, pH 7.4. Specific antisera was eluted from the columns with 0.2 M glycine–HCl, pH 2.2, dialysed against water, lyophylised and then redissolved at approximately 1 mg/ml in 0.02 M sodium dihydrogen orthophosphate, 0.15 M NaCl, pH 7.4 and stored at −20°C.

The anti-Type IV sera was used without passage through an immunoadsorbent column.

2.3. *Passive haemagglutination assay*

Antisera were tested for activity using a passive haemagglutination assay. The cells were coated with different collagen types using the tannic acid procedure described by Herbert [8]. A suspension of coated cells (2%, 0.05 ml) was incubated overnight at 4°C with 0.05 ml of a doubling dilution of antisera. Cross reactivity of the antisera was tested by titrating the anti-Type I, III and IV sera against Type I, III and IV collagen coated cells.

2.4. *Tissue staining*

The *extensor carpi radialis* muscle with attached tendon from a bullock was snap frozen with isopentane cooled by liquid nitrogen. 6–8 μm sections were cut in a cryostat and dried onto glass microscope slides.

Transverse sections of tendon and muscle were cut and stained initially with the specific rabbit anti-Type I, III, or IV collagen sera or normal rabbit serum as a control. Antisera were diluted to give a satisfactory staining intensity. The staining was carried out at 20°C for 30 min. The slides were washed for 1 h with 0.02 M sodium dihydrogen orthophosphate, 0.15 M NaCl pH 7.6 and then dried. The slides were subsequently stained with fluorescein conjugated anti-rabbit IgG (Burroughs Wellcome) for 30 min then washed as above for 2 h. As an additional control one set of slides was incubated for 30 min with unconjugated anti-rabbit IgG and washed for 1 h before being stained with the fluorescein-conjugated anti-rabbit IgG as above.

3. Results

3.1. *Collagen types*

Figure 1 shows the SDS–polyacrylamide gel electrophoresis patterns demonstrating the different mobilities

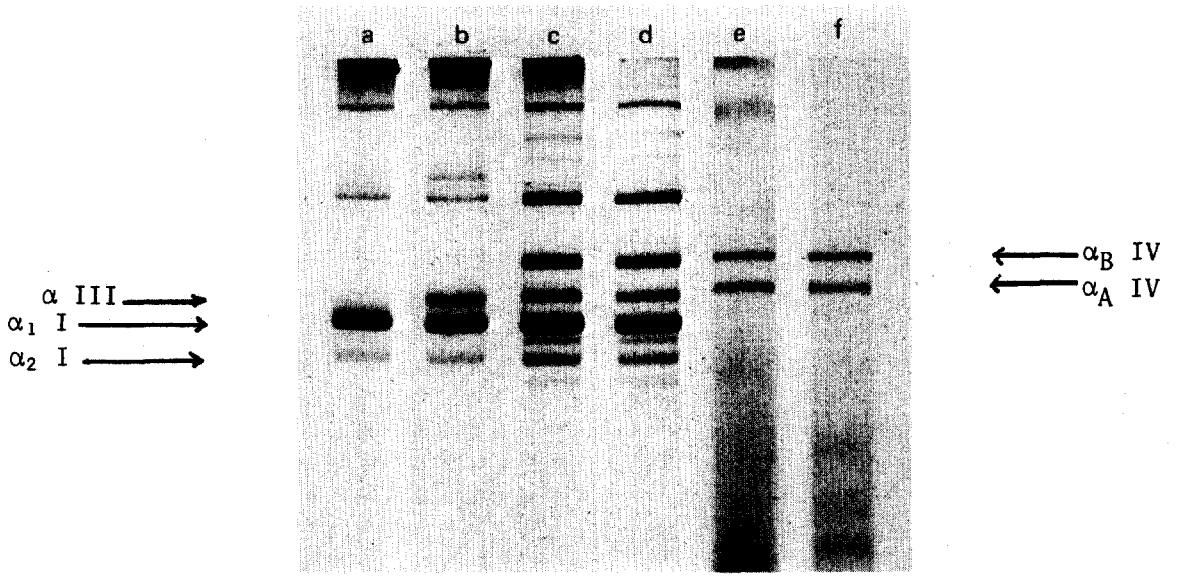

Fig.1. SDS–Polyacrylamide gel electrophoresis patterns of Type I, III and IV collagen from pepsin solubilized bovine intramuscular collagen. (a) and (b) show a mixture of Types I and III without and with mercaptoethanol (ME), respectively; note appearance of III following cleavage of the intra-molecular disulphide bonds with ME. (c) and (d) show Types I and IV without and with ME, respectively. (e) and (f) show the purified Type IV from human placenta without and with ME. Note absence of any effect of ME on the Type IV bands.

of Types I, III and IV collagens isolated from muscle. For comparison Type IV from human placenta is also shown.

3.2. *Purification of antibodies by immunoabsorption*

Following purification of the antibodies by affinity chromatography their specificity was checked by employing the passive haemagglutination assay. No cross-reactivity of the antisera was detected at the lowest antisera dilution used (1/4).

Agglutination titres of the antisera used for immunofluorescence ranged from 1/64 to 1/1024.

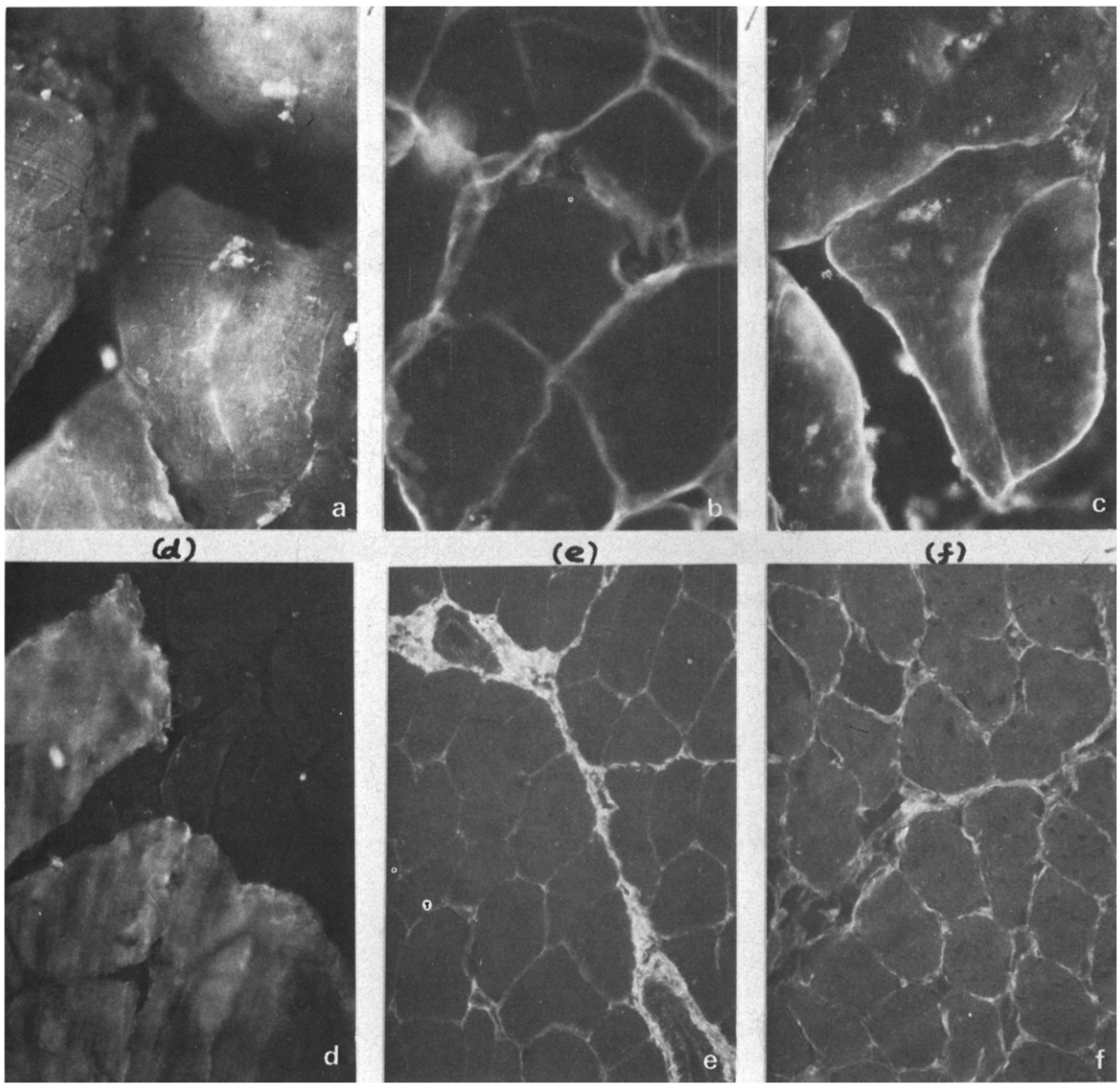

Fig. 2. Immunofluorescence staining of tendon (a–c) and muscle (d–f). (a) shows the overall staining of the tendon bundles with anti-Type I collagen whereas b and c show staining of the endotendineum only with anti-Type III and IV, respectively. Anti-Type I stains only the epimysium in muscle (d) whereas anti-Type III stains predominantly the perimysium with faint staining of the epimysium and endomysium (e). Anti-Type IV staining in muscle is mainly confined to the endomysium with very slight staining of the perimysium (f).

3.3. *Basement membrane Type IV collagen*

Antibodies to the collagen isolated from human placenta produced intense staining of anterior lens capsules, capillaries and glomerular basement membrane. Antibodies to Types I and III failed to elicit a staining reaction with isolated human glomerular basement membrane and anterior lens capsule. No cross-reaction of the Type IV was shown with Types I and III antibodies. Together with the chemical characterization these findings support the proposal that this collagen is a basement membrane collagen.

3.4. *Tissue staining*

The purified antibodies were shown to be tissue-specific in their staining reactions on the tendon and muscle.

3.4.1. Tendon

Using the indirect immunofluorescence technique with antibodies to Type I, staining throughout the whole tendon was observed (fig.2a). However, when stained with Type III and Type IV antibodies the staining of tendon cross-sections was restricted to the fine sheaths around the fibre bundles, i.e., the endotendineum (fig.2b, c, respectively).

3.4.2. Muscle

The epimysium exhibited a similar staining pattern to the tendon, i.e., overall Type I staining with staining of the microfibril sheath by Type III and Type IV antibodies. Figure 2d shows the epimysium staining intensely with anti-Type I with little or no staining of the intra-muscular perimysium and endomysium.

The perimysium exhibited strong immunofluorescence with Type III but not with Type IV antibodies. The endomysium on the other hand exhibited strong staining with Type III and Type IV antibodies but not with Type I (fig.2e, f, d, respectively). The results of these staining reactions are summarised in table 1.

Table 1
Relative intensities of the indirect immunofluorescence on section of skeletal muscle and tendon

		Antibodies to collagen		
		Type I	Type III	Type IV
Muscle –	epimysium	+++	+	+
	perimysium	–	+++	–
	endomysium	–	++	++
Tendon –	bundles	++	–	–
	endo-tendineum	–	++	++

4. Discussion

A tissue-specific localization of three distinct polymorphic forms of collagen has been shown to exist in skeletal muscle by immunofluorescence staining. The basement membrane collagen isolated from intra-muscular collagen [4] possesses properties similar to those of basement membrane collagen (Type IV). However, unlike the Type IV isolated from anterior lens capsule which was reported to contain three identical α-chains [9] the intra-muscular collagen appears to possess two different α-chains. This molecular composition is similar to basement membrane isolated from placenta [4] and to the collagen recently isolated from foetal membrane by Burgeson et al. [10]. As Burgeson et al. [10] pointed out these chains could be from separate molecules or a single molecule with the configuration $[\alpha_B]_2\alpha_A$. On the other hand Chung et al. [11] reported a different configuration for placental membrane involving α_B plus a 55 000 mol. wt α-chain. It is, of course, possible that a family of basement membrane collagens exists.

The designation of the collagen isolated from muscle and placenta as basement membrane was therefore equivocal. By preparing antibodies to this collagen and demonstrating its specific immunofluorescent staining with tissues classified as basement membrane we have now demonstrated that this collagen is indeed a basement membrane collagen.

Having obtained collagen type-specific antibodies to Types I, III and 'IV', they were then used to determine the distribution of these three polymorphic forms of collagen in muscle and tendon. Their location throughout the muscle was found to be highly specific.

Type I collagen is confined mainly to tendon, the epimysium and to a lesser extent the perimysium. Type III exists mainly in the perimysium of muscle, but also occurs in the thin sheaths around the microfibrils within the tendon (endotendineum). A similar staining of the endo-tendineum with antibodies to a peptide from Type III was recently reported by Becker et al. [12].

The Type IV is confined to the endomysium thus confirming that the basement membrane only previously identifiable by electron microscopy is a collagenous basement membrane. The endo-tendineum sheaths also stained intensely with the antibody to Type IV.

The histological silver staining technique for fine reticulin fibres could be delineating the Type III fibres, or possibly the glycoprotein associated with Type III. The Type III generally exists as fine fibres and the co-existence of Types III and IV suggests that a delicate network of these reticular Type III fibres probably acts as a support for basement membranes. This type of structure with its associated highly hydrated glycosaminoglycan would be ideal where mobility around the muscle cell is necessary.

The cellular derivation of these different collagens in muscle is unknown. Fibroblasts are present throughout the intramuscular connective tissue and presumably synthesise most of the collagen, although the role of the muscle cells cannot be dismissed since in vitro studies have clearly demonstrated that myoblasts are capable of synthesising collagen [13]. In the case of Type IV, based on the analysis of a number of tissues, Chung et al. [11] suggest that the A chain is epithelial in origin, the B chain derived from smooth muscle cells and that the 55 000 mol. wt component is endothelial in origin. On the other hand if A and B chains of basement membrane make up one molecule they are presumably derived from one source. Clearly the origin, development and the precise functional role of these specifically located polymorphic forms of collagen in muscle is intriguing and requires further investigation.

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